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Alteration in Transcripting the Gene Encoding the δ-Opioid Receptor in Rat Brain is not Underlying the Development of Tolerance to [D-Ala^2,D-Leu^5] Enkephalin

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Abstract

Previous study has demonstrated that chronic treatment of [D-Ala^2, D-Leu^5] enkephalin (DADLE) induces profound down-regulation of delta opioid receptor in rat brain. We further examined whether this down-regulation of receptor was due to a decrease in the transcription of gene encoding delta-opioid receptor (DOR-1). Rats received daily i.c.v. injection of DADLE for 1 , 3, or 5 days and developed significant tolerance to the antinociceptive effect of DADLE after one-day treatment. We measured the level of mRNA in rat brain tissues using in situ hybridization. No significant changes in the mRNA levels of the cortex, striatum, hippocampus, and thalamus on any examined days were found as compared to those of rats received sham operation only. There is only a transient decrease of DOR-1 mRNA level in midbrain region that occurred after a three-day treatment. Thus, the result of this study did not suggest that alteration in transcription of gene-encoding delta-opioid receptor was responsible for the down-regulation of delta-opioid receptor associated with the development of tolerance to DADLE.

Key Words: DADLE, DOR-1, in situ hybridization, rat brain

Introduction

The mechanisms underlying the development of tolerance to opioid are not clearly understood yet. Down regulation of opioid receptor has long been suggested as one possible mechanism. Although, previous studies of the chronic treatment of morphine did not constantly demonstrate decrease in the opioid receptor number or activity (4, 9, 12, 17, 22), chronic treatment of opioid agonists more selective for one of the subtype opioid receptor were successfully in showing a significant downregulation in the subtype opioid receptor. For instance, chronic treatment of PL017, a selective μ opioid receptor agonist, induced a downregulation of μ-opioid receptor number as well as a decrease in the coupling efficacy between the receptor and G-protein (19, 21); chronic treatment of agonists selective for δ-opioid receptor, such as [D-Ala^2, D-Leu^5]enkephalin (DADLE) and [D-Pen^2, D-Pen^5]enkephalin (DPDPE), induced a significant reduction of δ-opioid receptor number in rat brains (18, 20). In particular, daily intra-ventricular injection of DADLE in rats induced a drastic decrease in the δ-opioid receptor number in various brain regions, including the cortex, the striatum and the midbrain (20). The down-regulation of the δ-opioid receptor correlates well with the development of tolerance to the antinociceptive effect of DADLE. In the cortex, this down regulation occurred after a three-day treatment of DADLE. However, in the midbrain, a one-day treatment is sufficient to induce considerable loss of δ-opioid receptor number. There are at least two possible mechanisms responsible for such down-regulation of δ-opioid receptor. The first mechanism is an increase in internalization of δ-opioid receptors into lysosomal compartment thereby reducing the number of receptor in the neuronal membrane. This mechanism had been suggested by the previous study

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on the neuroblastoma cell line (13). Alternatively, a decrease in the transcription of the gene encoding the δ-opioid receptor may decrease the production of the É'-opioid receptor protein. The test of this possibility requires quantification of the mRNA of δ-opioid receptor gene in brain tissues using ribonucleotide or deoxyribonucleotide probes complementary to the gene sequence encoding δ-opioid receptor. By taking advantage of the cloned δ-opioid receptor, namely the DOR-1, the abundance of DOR-1 mRNA and its regional distribution in rat brain tissues have been clearly shown by northern blotting study and in situ hybridization study (6-8, 14, 15). Therefore, we used oligonucleotide-directed in situ hybridization to determine whether chronic treatment of DADLE would induce a reduction in transcripting DOR-1 in various brain regions of rat brain.

Materials and Methods

Animals Preparation

Male Sprague-Dawley rats weighing 200-250 gm were used in this study. All animals were provided food and water, and maintained on a twelve hours light/dark schedule. Rats were implanted with a stainless steel cannula into the left lateral cerebroventricle according to the coordinates: P 1.0 mm, L 1.25 mm, V 4-5 mm using bregma as zero. After the rats had recovered from the operation (3 days), DADLE was administered intra-cerebroventricularly (i.c.v) to determine the AD_{50} dose by the tail-flick antinociceptive response according to the up-down method described by Dixon (5). Afterwards, the animals were chronically treated with DADLE by daily administration of the peptide at 09.00 and 17.00 h starting with 10 μg i.c.v. dose on day 1, followed by a daily increase of the dosage by 10 μg, ending at 50 μg on day 5 of the treatment. After chronic DADLE treatment for 1, 3 or 5 days, the AD_{50} values of DADLE to elicit the antinociceptive response were re-determined on day 6 and rats were sacrificed after the tail-flick test. For acute DADLE treatment, rats were injected i.c.v. with 10 μg DADLE 20 min. prior to sacrifice.

Antinociceptive Assay

The tail-flick assay used was a modified method described by D'Amour and Smith (3). Heat from a high intensity lamp was focused onto a defined blackened area near the end of the tail of the rat. The latency for the rat to flick its tail away from the noxious heat stimulus was recorded before and after drug administration. The assays were performed at the peak effective time of DADLE.

Table 1. Alteration in the Antinociceptive Potency of DADLE during Chronic i.c.v. DADLE Treatment

Group	DADLE AD50(μg) Before treatment	DADLE AD50(μg) After treatment	Degree of tolerance
1 day	5.4±0.8	9.9±1.3	1.8
3 days	5.5±0.9	17.2±1.5	3.1
5 days	5.7±0.9	32.6±2.5	5.7

Values are means±S.E.M.. Each group contained more than six rats. Degree of tolerance was calculated from the ratio of (AD50 after treatment)/(AD50 before treatment).

In Situ Hybridization

The brain tissues were rapidly frozen in 2-methylbutane/methanol/dry ice bath and stored at -70°C. The tissues were cut into 16 μm slide tissues using a cryostat and mounted in gelatin-coating glass slide. The tissues were fixed in 4% paraformaldehyde and dried in ethanol. The hybridization was performed using a 51-bp oligonucleotide probe complementary to the 5' prime end. Its sequence was as follows: 5'GGCTCCC GGCGACCCCGACGCATTG GCGCCCGCGCTGGGGAAGGCGCTGGG'3. The oligonucleotide probe was labeled with S^{35}αATP using 3-end labeling transferase kit (Dupont). The hybridization buffer contained 50% formamide, 10% dextran, 4 x Denhart's solution, 100 μg/ml sperm DNA, and 1 X SSC. The tissues were incubated at 40°C for 16 hours and then washed in 1 x SSC for 10 minutes at room temperature, 0.1 x SSC at 55°C for 10 minutes and 0.1 X SSC at room temperature for 20 minutes. The tissues were exposed to x-ray film for four weeks. The optic density was counted using computerized image analyzer. In each x-ray film, one slide pasted with standard radio-labeled makers was also exposed side by side with brain tissues to calibrate the radioactivity (nCi/mg) of each examined area from optic density.

Results

Rats developed significant tolerance to the antinociceptive effect of DADLE after one day treatment of DADLE, manifested by the apparent increase of the AD50 dose of DADLE (Table 1). In particular, the increase in the AD50 of DADLE rose drastically after a 3-day treatment, confirming the establishment of tolerance to DADLE. In situ hybridization in normal rats demonstrated an uneven distribution of mRNA of DOR-1 throughout entire

brain. The highest density of DOR-1 mRNA was in the dentate gyrus of the hippocampus and the midbrain area (Fig. 1). The cortex, striatum and thalamus contained the moderate abundance of DOR-1 mRNA. The comparison of the level of mRNA in several brain regions between control and DADLE-treated group showed only a transient 38 % decrease at midbrain region of the DADLE-treated group at day 3 after the beginning of DADLE treatment. There was no significant difference between these two groups of rats on any other examined days and at any other examined brain regions (Table 2).

Discussion

The present study demonstrated a specific regional distribution of DOR-1 mRNA, which was quite similar to those of previous study using cRNA probe-directed in situ hybridization (8, 14, 15). This DOR-1 mRNA distribution in general correlated to the localization of δ-opioid receptor demonstrated by receptor ligand binding autoradiography (15, 16).

In contrast to δ-opioid receptor ligand binding study, chronic treatment of DADLE did not produce drastic or persistent alteration in the abundance of DOR-1 mRNA in any examined brain regions. Although there was a transient decrease of DOR-1 mRNA in the midbrain of DADLE group, the magnitude and persistency of this decrease did not match with the loss of δ-opioid receptor number revealed by previous ligand binding study (20). It seems that this observation did not support the idea that alteration in transcription of the δ-opioid receptor gene was responsible for the down-regulation of δ-opioid receptor in rats developed tolerance to DADLE. Rather, mechanisms such as increase in internalization of receptor, decreased in the translation of mRNA, alteration in the post-translation modification or others may be involved. However, before making any definitive conclusion for the present study, there are two limitations of the in situ hybridization assay that should be taken into account. Firstly, the northern blotting analysis of our preliminary study (data not shown) and by other reports have shown that multiple transcripts of DOR-1 are present in the rat brain tissues (2, 6, 7). At least six transcripts with different base pairs are found, implying that DOR-1 may have several splicing forms of mRNA. The exact sequence of each transcript and its role on the expression of δ-opioid receptor are not fully elucidated yet. Since the probes used in hybridization can not distinguish each transcript of DOR-1 from others, the result of in situ hybridization can only reflect the total abundance of these DOR-1 transcripts. Thus, we can not exclude the possibility that alteration in one of these different transcripts is present. To test this possibility,

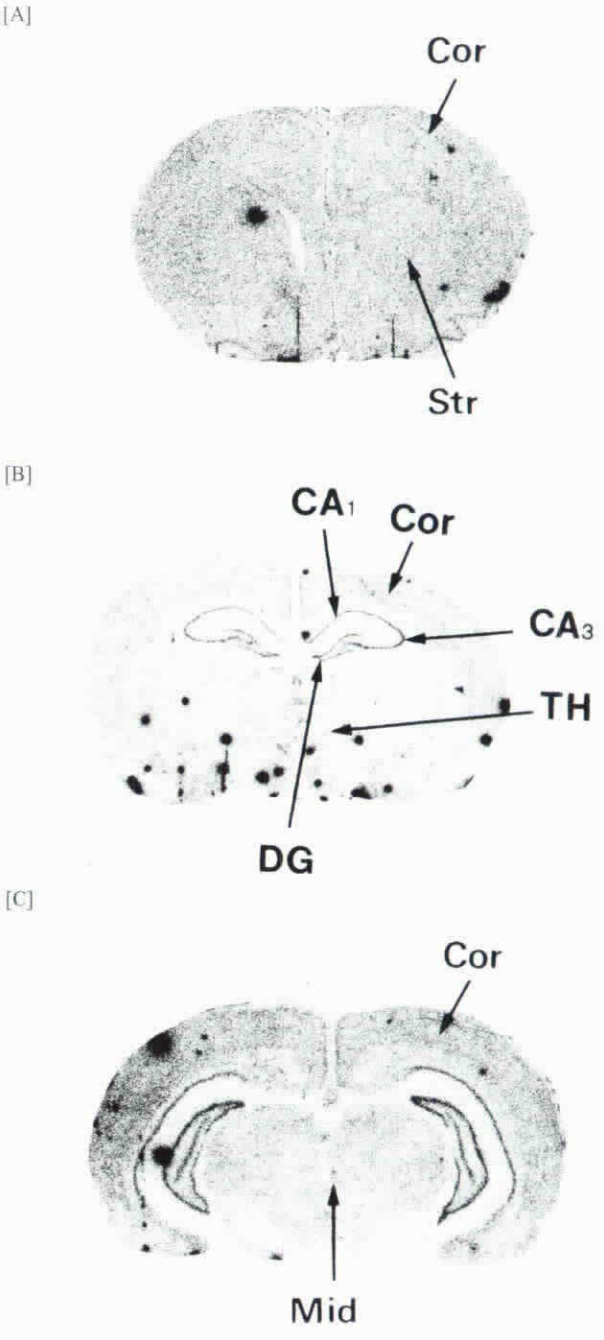

Fig. 1. The regional distribution of DOR-1 mRNA in normal rat brain. [A] Cor: cortex, Str : striatum; [B] DG: dentate gyrus, TH: thalamus; [C] Mid: midbrain.

quantification of DOR-1 mRNA by northern blotting or by reverse transcriptase-polymerase chain reaction (RT-PCR) could be the technique of choice. Secondly, the result of this in situ hybridization only represent the level of DOR-1 mRNA under steady-state condition and does not necessarily reflect the rate of production or degradation of the mRNA. It remains possible that alteration in these two parameters could potentially affect the efficacy of mRNA translation.

Table 2. Effects of Repeated DADLE Treatment on the Level of DOR-1 mRNA in Different Rat Brain Regions. (unit: nCi/mg)

Regions	Control (n=6)		1 day (n=7)		3 days (n=6)		5 days (n=7)	
	Rt	Lf	Rt	Lf	Rt	Lf	Rt	Lf
Cortex	1.16±0.13	1.34±0.16	1.13±0.12	1.14±0.18	1.08±0.06	1.23±0.03	1.26±0.24	1.30±0.24
Striatum	1.21±0.13	1.30±0.16	1.09±0.08	1.17±0.10	0.97±0.06	1.04±0.05	1.16±0.18	1.34±0.10
CA1	1.40±0.14	1.63±0.17	1.24±0.13	1.30±0.17	1.26±0.07	1.36±0.05	1.20±0.25	1.31±0.23
CA3	1.53±0.19	1.71±0.30	1.25±0.15	1.23±0.15	1.28±0.12	1.38±0.03	1.23±0.28	1.50±0.20
DG	2.45±0.28	2.50±0.33	2.29±0.05	2.37±0.15	2.24±0.28	2.36±0.36	2.24±0.28	2.36±0.36
Thalamus	1.11±0.15	1.26±0.10	1.06±0.12	1.17±0.15	0.95±0.05	1.00±0.05	0.91±0.20	1.10±0.18
Midbrain	2.19±0.36	2.21±0.36	1.96±0.15	1.75±0.09	1.40±0.14*	1.35±0.08*	1.97±0.13	2.06±0.15

Values are reported as mean±S.E.M. DG: dentate gyrus
*Signficantly different to that from control group ($p<0.05$, two way ANOVA with post-hoc Scheffe's test)

For instance, decrease of both production and degradation of mRNA may mean a deceleration in the translation rate of mRNA, hence the production of δ-opioid receptor may be reduced. On the contrary, increased production and degradation of mRNA means acceleration in the translation of mRNA. Under both conditions, the steady-state mRNA level may be maintained at similar and constant level. However, it is more feasible to test this possibility in vitro on the cell culture rather than using intact animal tissues.

Interestingly, δ-opioid receptor expression also can be altered by chronic treatment of ethanol, though the results are quite contradictory. An in vivo study on mice showed a down-regulation of the δ-opioid receptor induced by the treatment of ethanol (10). But no report has shown a decrease in the abundance of DOR-1 mRNA in brain. On the contrary, the in vitro study on the neurobalsoma x glioma cell, the NG108-15 cell, showed an up-regulation of the δ-opioid receptor (1). Subsequent studies with northern blotting shown that increase in the abundance of DOR-1 mRNA might be responsible for such ethanol-induced up-regulation of δ-opioid receptor (2, 11). In these studies, the northern blotting also exhibited multiple transcripts in the NG 108-15 cells and chronic treatment of ethanol increased the abundance of each transcript with similar magnitudes. However, an earlier study showed that down-regulation of δ-opioid receptor in NG 108-15 cells occurred in the presence of actinomycin D, but not in the presence of cycloheximide. Hence normal protein synthesis, but not DNA transcription, is required for the alteration of the expression of δ-opioid receptor (1). The causes of the disparity between these results may be complicated but the most plausible implication is that transcription of DOR-1 is not necessary responsible for the alteration of the expression of δ-opioid receptor on neuronal membranes.

In summary, we demonstrated here that chronic DADLE treatment in rats induced apparent tolerance to this opioid peptide but did not produce significant and persistent change in the quantity of DOR-1 mRNA in various brain regions. Future investigations may include using techniques other than in situ hybridization to quantify the rat brain mRNA in more detail to rule out the possible involvement of DNA transcription in the down-regulation of the δ-opioid receptor. Alternatively, it may be necessary to focus on the process involving in the receptor expression, which are downstream from the DNA transcription.

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